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Production of Types A and B Spores of *Clostridium botulinum* by the Biphasic Method: Effect on Spore Population, Radiation Resistance, and Toxigenicity

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Spores of three strains each of type A and type B *Clostridium botulinum* were produced both by a biphasic (solid medium overlaid with an aqueous phase) and by a "conventional" (deep broth culture) procedure. Sporogenesis by the biphasic system was more rapid, convenient, and economical, and yielded as many or more heat-resistant (80 C, 10 min) spores per milliliter as by the conventional technique. Of several aqueous phases [thiamine-hydrochloride, yeast extract, $(NH_4)_2SO_4$] tested with strain 62A, the highest spore colony counts were obtained with 2.0% $(NH_4)_2SO_4$. The six strains formed maximum spore numbers in 5 to 6 days of incubation. Spores produced by the two methods had essentially equal radiation resistances (D and lag values), and their subcultures gave similar toxin titers (LD_{50} values).

Our program of inoculated pack studies designed to determine the minimal radiation sterilization dose for foods requires a minimum of 100 ml of spore suspension containing 10^8 spores/ml of each of 10 strains of *Clostridium botulinum*. As reported elsewhere (1), our "conventional" (deep broth culture) method for generating such quantities of spores necessitates the build-up of inoculum volumes by successive 10% transfers for five consecutive days and finally inoculating a total of 3,400 to 9,000 ml of culture medium and incubating for 7 to 12 days, depending on the strain used. Such large quantities of active cultures are relatively hazardous to handle, occasionally produce low spore numbers or contamination, or both, and entail excessive manipulations and equipment (e.g., 17 to 45 centrifuge bottles per strain). The preparation and harvesting of 10 strains of *C. botulinum* spores require a minimum of 6 weeks of laboratory effort; hence, this service was performed on a contract basis. Consequently, it would be desirable to produce spores by a more rapid, convenient, and economical technique without decreasing their radiation resistance and capacity to form toxin in growth cultures.

Bruch et al. (4), employing a biphasic procedure, obtained 10^7 to 5×10^8 spores/ml (with 95% sporogenesis) with two strains of type E *C. botulinum*; the liquid phase of their system was 500 ml of distilled water. Anellis and Rowley (1) investigated this method for the spore production of *C. botulinum* strain 62A (type A). It was observed that the composition of the liquid phase influenced the percentage of sporulation and the heat-stable (80 C for 10 min) spore yield. Of five liquid phases examined, aqueous thiamine-hydrochloride (0.0001%) gave the highest spore numbers (6.6 $\times 10^7$ spores/ml with 80% sporogenesis in 500 ml of liquid after 5 days of incubation at 37 C). The spore crops also appeared to be microscopically cleaner (fewer vegetative forms, sporangia, and debris) than those secured by the conventional technique. Hence, the biphasic system was investigated more closely as a possible means of replacing the conventional production of spores. The present communication examines the effect of several additional liquid phases on sporogenesis, and compares the radiation resistance of, and toxin-forming capability of cultures derived from, spores of three type A and three type B *C. botulinum* strains produced by the biphasic and conventional procedures.

MATERIALS AND METHODS

Test organisms. Three representative strains of *C. botulinum* type A (33A, 36A, 62A) and three of type B (9B, 40B, 41B) were selected from our collection.

Spore production: (i) conventional method. Spores were prepared by the technique previously described (1), using the Trypticase peptone (TP) broth.

(ii) Biphasic method. The organisms were propagated in broth composed of (w/v) 5% BBL Trypticase, 0.5% peptone, 0.125% K_2HPO_4, and adjusted to *p*H 7.5 with 5 N KOH before autoclaving. Filter-sterilized $NaHCO_3$ was added to a final concentration of 0.075% prior to inoculation. The inoculation sequence is shown in Table 1. For sporulation, the agar phase was of the same composition, except that the $NaHCO_3$ was omitted and 0.1% BBL yeast extract and 3% agar were added. The agar phase (1,000 ml) was prepared on the day of use in 2,800-ml Fernbach flasks, autoclaved, cooled rapidly to 20 to 30 C, and overlaid aseptically with various sterile liquid phases. The flask headspace was flushed with sterile nitrogen for 5 min before and after inoculation. Incubation was at 30 C, and observations were made periodically for heat-resistant (80 C, 10 min) and germinated spores.

Enumeration of spores. Samples taken periodically of both conventional and biphasic cultures were heat-shocked (80 C, 10 min) and decimally diluted in sterile distilled water; 1.0-ml volumes were inoculated into triplicate tubes (12 by 200 mm) containing 0.3 ml of filter-sterilized 5% NaHCO and agar medium (5% BBL Thiotone, 0.05% sodium thioglycolate, 0.85% Ionagar, pH 7.2) added. Uniform mixing of the spores was obtained by the swirling motion of the agar with the tube contents during pouring. The tubes were stratified after solidification with about 1.5 cm of the same medium and incubated at 30 C for 36 to 48 hr, and the macrocolonies in the triplicate tubes were averaged.

Microscopic observation. Dry smears of cultures were stained with 0.5% aqueous methylene blue, and the percentage of germinated (stained) spores was estimated by counting 200 cells under phase-contrast optics.

TABLE 1. *Inoculation sequence of Clostridium botulinum for the preparation of spores by the biphasic method*

Inoculation sequence	Incubation at 30 C (hr)
Spore stock[a] (5 ml)	
↓	
20 ml broth[b] (5 ml)	24
↓	
20 ml broth[b] (5 ml)	4
↓	
20 ml broth[b] (25 ml)	4
↓	
Biphasic system[b]	Up to 11 days

[a] Brain liver heart (Difco); heated at 80 C for 10 min and cooled rapidly to 20 to 30 C.

[b] See Materials and Methods.

Harvesting of spore crops. The spores were harvested by three successive centrifugations and resuspensions in 0.067 M Sorensen phosphate buffer, *p*H 7.0, at 2,520 × *g* for 20 min at 2 to 5 C and stored in 100 ml of buffer at 2 to 5 C until used.

Radiation resistance. Duplicate tubes (16 by 150 mm) containing 5.5 ml of heat-shocked (80 C, 10 min) spore suspension were sealed under vacuum (380-mm pressure) in a tube sealer designed for controlling different gaseous atmospheres at various pressures (O. P. Snyder, Jr., Ph.D. thesis, Univ. of Massachusetts, Amherst, 1969) and were irradiated with Co^{60} gamma rays at -30 ± 3 C in increments of 0.1 mrad from 0 to 1.6 mrad. The irradiated tubes were thawed overnight at 2 to 5 C, thoroughly mixed on a Vortex mixer, and opened aseptically; the survivors were counted as above, but without additional heating. D values (dose for a 10-fold decrease in spore level) were determined from slopes of survival curves plotted on semilogarithmic paper. The lag (or shoulder) of the curves was obtained by the graphical method of Durban and Grecz (5).

Titration of toxin. One-milliliter samples of heat-shocked (80 C, 10 min) spore stocks (10^8 spores/ml) were inoculated into tubes (20 by 150 mm) containing 20 ml of cooked meat medium (Difco), incubated at 30 C for 14 days, and centrifuged. The supernatant fluid was appropriately diluted in gelatin-phosphate (0.2% gelatin, 0.4% Na_2HPO_4, *p*H 6.2, adjusted with N HCl), and 0.5-ml quantities of each dilution were injected intraperitoneally into each of 10 unprotected white mice (15 to 20 g weight) and into each of 10 mice protected with *C. botulinum* type-specific antitoxin. Observations for typical botulinal death were made for 4 consecutive days.

LD_{50} values were computed by the equation $LD_{50} = t_u + d/2 - d\sum_{i=i}^{u} P_i$ (2), where t_u is the highest dilution of toxin which caused mouse death, d is the dilution increments used, u is the number of toxin dilution levels below the minimal lethal dilution, and P_i is the percentage of live mice at each dilution increment between 0 and 100%.

RESULTS AND DISCUSSION

Effect of liquid phase composition on sporogenesis. Of several liquid phases (500-ml quantities) previously examined with the biphasic system for the production of *C. botulinum* 62A spores, Anellis and Rowley (1) obtained highest yields with aqueous thiamine-hydrochloride (0.0001%). Hence, this volume (a liquid-solid ratio of 1:2) was compared with 250 ml of the vitamin solution (a liquid-solid ratio of 1:4). Heat-resistant spore count data indicated that the 1:4 ratio gave higher spore yields than the 1:2 ratio for periods of incubation between 3 and 11 days (Table 2). Tyrrell et al. (7) observed a similar liquid-solid ratio effect with other organisms. Therefore, liquid phases of 0.1% yeast extract (BBL) and 2.0 and 0.5% $(NH_4)_2SO_4$, using a liquid-solid volume of 1:4, were then compared with the 250-ml thiamine-hydrochloride phase.

Maximum numbers of spores occurred with 2.0% $(NH_4)_2SO_4$. Similar stimulatory sporulation effects by ammonium and sulfate ions were reported by Leifson (6) for *C. botulinum* and *C. tetani*, by Schmidt (Bacteriol. Proc., p. 44, 1960) for *C. botulinum*, and by Brown et al. (3) for putrefactive anaerobe 3679 (PA3679).

Highest spore populations of strain 62A *C. botulinum* were observed on the sixth day of incubation with only 10% of germinated spores. On the seventh day the spore colony count had decreased from 12.0×10^8/ml to 8.3×10^8/ml. The decline continued up to the eleventh day with a concomitant rise in the concentration of germinated spores (38%).

Sporogenesis of C. botulinum strains. When 250 ml of 2% $(NH_4)_2SO_4$ was used as the standard liquid phase for eliciting sporogenesis of five other strains of *C. botulinum* (33A, 36A, 9B, 40B, 41B), the spore populations attained were as impressive as with strain 62A (Table 3). The spore colony counts reached 10^8 to 10^9/ml in 4 to 6 days of incubation, depending on

TABLE 2. *Spore production of Clostridium botulinum strain 62A in various biphasic systems*

Incubation at 30 C (Days)	Liquid phase		Heat-resistant spore count/ml[b] ($\times 10^8$)	Germinated spores[c] (%)
	Substance	Concn[a] (%)		
3	Thiamine-hydrochloride[d]	0.0001	1.9	<1
	Thiamine-hydrochloride	0.0001	4.5	<1
	Yeast extract	0.1	4.2	<1
	$(NH_4)_2SO_4$	2.0	2.9	<1
	$(NH_4)_2SO_4$	0.5	4.3	<1
4	Thiamine-hydrochloride[d]	0.0001	6.5	<1
	Thiamine-hydrochloride	0.0001	8.7	<1
	Yeast extract	0.1	10.0	<1
	$(NH_4)_2SO_4$	2.0	9.7	<1
	$(NH_4)_2SO_4$	0.5	8.8	<1
5	Thiamine-hydrochloride[d]	0.0001	4.7	7
	Thiamine-hydrochloride	0.0001	8.8	10
	Yeast extract	0.1	8.9	10
	$(NH_4)_2SO_4$	2.0	10.0	10
	$(NH_4)_2SO_4$	0.5	8.0	6
6	Thiamine-hydrochloride[d]	0.0001	3.2	17
	Thiamine-hydrochloride	0.0001	8.8	11
	Yeast extract	0.1	9.1	12
	$(NH_4)_2SO_4$	2.0	12.0	10
	$(NH_4)_2SO_4$	0.5	9.3	9
7	Thiamine-hydrochloride[d]	0.0001	3.1	25
	Thiamine-hydrochloride	0.0001	7.6	21
	Yeast extract	0.1	8.9	27
	$(NH_4)_2SO_4$	2.0	8.3	26
	$(NH_4)_2SO_4$	0.5	5.9	26
11	Thiamine-hydrochloride[d]	0.0001	1.1	43
	Thiamine-hydrochloride	0.0001	4.3	42
	Yeast extract	0.1	2.9	48
	$(NH_4)_2SO_4$	2.0	4.7	38
	$(NH_4)_2SO_4$	0.5	4.0	50

[a] Aqueous solution (w/v).
[b] Heated at 80 C for 10 min.
[c] Germinated spores per total spores ×100.
[d] Five hundred-milliliter solution, or a liquid-agar ratio of 1:2. The remainder of the liquid phases have a 250-ml volume, or a liquid-agar ratio of 1:4.

TABLE 3. *Spore production of Clostridium botulinum strains in biphasic culture*[a]

Incubation at 30 C (Days)	Strain	Heat-resistant spore count/ml[b] ($\times 10^8$)	Germinated spores[c] (%)
3	33A	5.2	<1
	36A	3.8	<1
	62A	8.4	<1
	9B	2.7	<1
	40B	4.9	<1
	41B	1.4	<1
4	33A	8.2	<1
	36A	6.6	<1
	62A	14.1	<1
	9B	5.7	<1
	40B	6.8	<1
	41B	2.5	<1
5	33A	10.1	<1
	36A	11.1	<1
	62A	12.0	10
	9B	12.0	<1
	40B	9.0	<1
	41B	3.4	1
6	33A	10.9	5
	36A	10.8	9
	62A	12.4	14
	9B	7.5	
	40B	7.0	6
	41B	3.5	1
7	33A	10.3	5
	36A	7.5	15
	62A	10.8	23
	9B	4.9	
	40B	5.8	5
	41B	2.4	5
10	33A	6.4	8
	36A	3.7	26
	62A	4.9	36
	9B	2.8	5
	40B	2.2	23
	41B	2.3	8

[a] Liquid phase: 250 ml of 2.0% $(NH_4)_2SO_4$.
[b] Heated at 80 C for 10 min.
[c] Germinated spores per total spores $\times$ 100.

TABLE 4. *Comparative spore production of Clostridium botulinum strains by the biphasic and conventional methods*

Strain	Expt no.	No. of spores/ml[a] by biphasic method[b] ($\times 10^8$)[d]	Conventional method[c]		
			No. of spores/ml[a] ($\times 10^8$)[d]	Vol of broth (ml)	Incubation at 30 C (days)
33A	1	62.0	27.0	4,000	7
	2	10.1	20.0	4,000	7
	3	8.2	26.0	4,000	7
36A	1	7.9	15.0	9,000	12
	2	10.8	1.2	9,000	12
	3	4.5	20.0	9,000	12
62A	1	23.6	9.8	4,000	7
	2	12.0	10.0	4,000	7
	3	8.1	13.0	4,000	7
9B	1	29.0	1.2	6,000	11
	2	7.5	5.6	6,000	11
	3	7.9	16.0	6,000	11
40B	1	24.1	5.8	4,000	8
	2	7.0	2.8	4,000	8
	3	8.6	2.1	4,000	8
41B	1	8.1	1.8	3,400	11
	2	3.5	4.3	3,400	11
	3	9.3	3.4	3,400	11

[a] Concentrated to 100 ml of stock spore suspension.
[b] Three Fernbach flasks per strain (750 ml of liquid), incubated for 6 days at 30 C; 250 ml of aqueous 2.0% $(NH_4)_2SO_4$; agar phase as in Materials and Methods.
[c] Five per cent Trypticase (BBL), 0.5% peptone (Difco), 0.05% sodium thioglycolate, pH 7.2.
[d] Heated (80 C, 10 min) spore counts.

TABLE 5. *Comparative radiation resistance of Clostridium botulinum spores produced by the biphasic and conventional methods*

Strain	Biphasic method (B)		Conventional method (C)		B/C	
	D (mrad)[a]	Lag (mrad)[b]	D (mrad)	Lag (mrad)	D	Lag
33A	0.13	0.30	0.10	0.32	1.3	0.9
36A	0.13	0.24	0.13	0.23	1.0	1.0
62A	0.14	0.28	0.12	0.28	1.2	1.0
9B	0.13	0.25	0.12	0.27	1.1	0.9
40B	0.10	0.32	0.10	0.31	1.0	1.0
41B	0.11	0.28	0.10	0.28	1.1	1.0

[a] Negative reciprocal of the slope of the exponential portion of the survival curve.
[b] Obtained by the method of Durban and Grecz (5).

TABLE 6. *Comparative toxin titers in vegetative cultures derived from biphasically and conventionally produced Clostridium botulinum spores*

Strain	LD_{50} ($\times 10^{5}$)/ml[a] in fluid from	
	Biphasic spores	Conventional spores
33A	1.4	2.2
36A	2.0	1.5
62A	1.4	1.2
9B	4.4	4.7
40B	61	45
41B	88	82

[a] Computed by the equation $LD_{50} = t_u + d/2 - d\sum_{i=1}^{u} P_i$ (see Materials and Methods).

the strain, with an attendant low percentage of germinated spores. As incubation continued, the heat-resistant spore concentrations decreased and the yield of germinated spores increased.

In three separate experiments (Table 4), the spore yields obtained by pooling the liquid contents of three biphasic flasks after 6 days of incubation (750 ml of liquid, or four 250-ml centrifuge bottles) were considerably higher than our minimal needs (100 ml, 10^8 spores/ml), and in 9 of the 18 total trials were higher than the spore populations secured by the conventional procedure. The volume of broth required by the latter method to equal or surpass the concentration of biphasically produced spores varied from a minimum of 3,400 ml (17 centrifuge bottles) to a maximum of 9,000 ml (45 centrifuge bottles), and the periods of incubation varied from 7 to 12 days (plus an additional 5 days of inoculum build-up) instead of 6 (plus an additional 32 hr of inoculum preparation) depending on the strain used.

Radiation resistance. The rate of death of the spores of all six strains was unaffected by the method of spore production (Table 5). The D values ranged from 0.10 to 0.14 mrad for the biphasic spores and 0.10 to 0.13 mrad for the conventional spores. The lag of the survival curves was also unchanged by the two spore production methods (0.24 to 0.32 and 0.27 to 0.32 mrad, respectively).

Toxin titer. Regardless of the procedure of sporogenesis, the capabilities of spore-initiated cultures to produce high levels of toxin were essentially identical (Table 6). Four strains (33A, 36A, 62A, 9B) produced LD_{50} titers of about 10^{-5}, whereas the remaining two strains (40B, 41B) elaborated approximately 0.1-fold this quantity (LD_{50} values closer to 10^{-4}).

The present experiments demonstrated that the biphasic system is a more rapid, convenient, and economical means of obtaining *C. botulinum* spores than the conventional method used previously in our laboratory. Furthermore, there was no essential difference in the radiation resistance or the toxin-forming capability of the spores produced by the two techniques. Thus, spores elicited biphasically should provide a microbial index of safety for radiation-sterilized foods equivalent to that provided by conventionally formed spores.

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